Analysis of *Radix Bupleuri* (Chaihu) using Agilent 1200 Series LC Systems with Evaporative Light Scattering Detector

Application Note

Traditional Chinese Medicine

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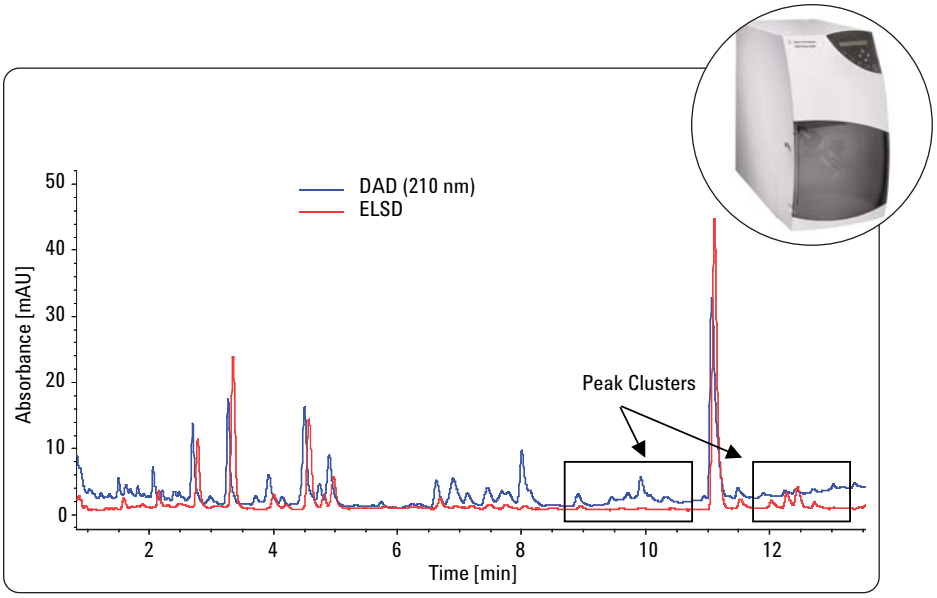

Abstract

This Application Note describes the development of conventional HPLC and Rapid Resolution LC (RRLC) methods for the analysis of *Buplerui* (Chaihu). A comparison of fingerprint chromatograms of different *Buplerui* samples showed different peak profiles of different TCMs collected from different places. The method for the Agilent 1200 Series Evaporative Light Scattering Detector (ELSD) was optimized to achieve maximum response. A quantitative method was developed to determine the six types of saikosaponins in different TCMs and their preparations.

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Introduction

Chaihu, the dried roots of the plants *Bupleurum Chinese DC* or *B. scorzonerifolium (Fam. Umbelliferae)* is one of the most popular Traditional Chinese Medicines (TCM). According to the description in the China Pharmacopeia[1], *B. Chinese* DC and *B. scorzonerifolium (Fam. Umbelliferae)* are known respectively as "Beichaihu" (Northen Chinese Thorowaz Root) and "Nanchaihu"(Southern Chinese Thorowas Root). Besides Beichaihu and Nanchaihu, there are other species that used as Chaihu in China. Radix bupleuri (Chaihu) is used in many multicomponent formulas in Chinese patent medicine. It can be used to treat illnesses such as influenza and fever.

Chaihu contains several kinds of components and saikosaponins are believed to be the most important with regard to the therapeutical effects. Saikosaponins have been determined previously in various species of Chiahu using HPLC with UV detection at low wavelengths such as 210 nm or below. At these wavelengths it is not possible to maintain a flat baseline when using gradients with organic solvents such as methanol. The drifting baseline may have some influence on quantitative analysis because the peaks might not be able to be integrated. Some of the saikosaponins in Chaihu are oleanane saponins and have UV absorbance only at low wavelengths. Other saikosaponins have dienes and can therefore be detected more easily by UV detection. Oleanane saikosaponins are unstable when subjected to high temperature or low pH conditions during Chaihu slice or HPLC sample preparation and turn into diene saponins.

Some research of Chaihu has been performed using LC/MS instrumentation. The detection limit or sensitivity is better but the instrumentation is more expensive and operators need to have knowledge of MS. There is currently a trend to analyze Chaihu using an evaporative light scattering detector (ELSD) and several papers have been published in Chinese scientific journals[2,3]. An ELSD is able to detect all analytes that are less volatile than the solvent. The baseline can be maintained stable regardless of the solvent's UV absorbance or the gradient conditions. Saikosaponins with one or two double bonds can be detected without discrimination. Some volatile components, which exhibit UV absorbance at low wavelengths, do not show response when using an ELSD and therefore do not interfere with integration of the saikosaponins during quantitative analysis.

Fingerprint chromatograms of Chaihu are important to help identify the origin. The components other than saikosaponins in this TCM also have different contributions to the therapeutic effects. To obtain holistic information about Chaihu, both a UV detector and an ELSD should be used in the application to detect the peaks from the saikosaoponins and from the other components.

According to the referenced literature, about 50 minutes are needed for conventional HPLC methods to separate several saikosaponins. To accelerate the analysis process, RRLC has recently attracted more and more attention. The results of RRLC maintain similar efficiency while reducing run time and cost. Two methods are used in this study to provide references for the different LC system configurations. The different profiles of the different samples are discussed and quantitative analysis gives ideas on the different amount of the saikosaponins in different samples.

Experimental

Standards and materials

6''-acetyl-SSa (3), 3''-acetyl-SSd (5) and 6''-acetyl-SSd (6) were isolated from the roots of Bupleuri Chinese in the laboratory. SSa (2) and SSd (4) were purchased from National Institute for the Control of Pharma-ceutical and Biological Products, Beijing, China. SSc (1) was purchased from National Pharmaceutical Engineering Center for Solid Preparation in Chinese Herbal Medicine (Jiangxi, China). Their structures were elucidated by comparison of their spectral data (UV, MS, 1H NMR, and 13C NMR) with literature values. The raw medicine was collected by the staff of the research group.

Equipment

An Agilent 1200 Series Rapid Resolution LC system was used for development of both conventional LC and RRLC methods. The system comprised the following modules:

- Agilent 1200 Series Binary Pump SL with Vacuum Degasser
- Agilent 1200 Series High-performance Autosampler SL
- Agilent 1200 Series Thermostatted Column Compartment SL
- Agilent 1200 Series Diode Array Detector SL with micro flow cell (2 µL volume, 3 mm path length)
- Agilent 1200 Series Evaporative Light Scattering Detector with standard nebulizer
- Agilent ChemStation software revision B.03.02 for system control, data acquisition and evaluation

Sample preparation

Finely powdered dried plant materials (0.5 g) were treated ultrasonically in 8 mL of methanol (containing 2 % pyridine) for 30 min and centrifuged for 10 min at 3,000 rpm. This procedure was repeated three times and the respective supernatants were combined and concentrated in a rotary evaporator. Methanol was used to dilute the concentrated solution ultrasonically and the volume was made up to exactly 10 mL. All solvents were filtered through a 0.22 µm nylon membrane prior to direct injection. Table 1 shows the different sample numbers and corresponding names.

Name	Sample
Bupleunum chinese	3
Bupleunum scorzonerifolium	12
Bupleunum smithii var.parvifolium	21
Bupleunum bicaule	25
Bupleunum rockii	27

Table 1
Names and sample numbers.

Conventional HPLC method

Solvent A: Water
Solvent B: Acentonitrile
Flow rate: 1 mL/min
Gradient: 0 min, 30 %B;
5 min, 40 %B;
25 min, 50 %B;
60 min, 100 %B
Injection volume: 15 µL
Column: ZORBAX SB C18,
4.6 x 250 mm,
5 µm particle size
Temperature: Ambient
UV detector: 210 nm, 248 nm,
standard flow cell
ELSD: Temperature: 40 °C
pressure: 55 psi
gain: 7
filter: 3s

RRLC method

Solvent A: Water
Solvent B: Acentonitrile
Flow rate: 0.8 mL/min
Gradient: 30 %B, 1 min;
40 %B, 5 min;
50 %B, 14 min,
100 %B
Injection volume: 2 µL
Column: ZORBAX SB C18,
3 x 50 mm, 1.8 µm particle size
Temperature: Ambient
UV detector: 210 nm, 248 nm,
micro flow cell
ELSD: Temperature: 40 °C
pressure: 55 psi
gain: 7
filter: 3s

Results and discussion

The first step in the study was to separate the saikosaponins standards. At the same time the ELSD parameters were optimized so that most of the saikosaponins showed best sensitivity although it was not possible to achieve maximum response for all analytes. The temperature and gas pressure of the ELSD were the main parameters that needed to be optimized. A temperature of 40 °C and a pressure of 55 psi gave the best results. These values were used in the final method for further analysis. The complete methods for conventional HPLC and RRLC are shown in the experimental section.

Figure 1 shows the chromatograms of the six saikosaponins from the conventional HPLC analysis using ELSD and UV detection. The UV channel detected more peaks and the baseline showed some drifting. The solvents used in sample preparation exhibited response in the UV channel but showed no signal in the ELSD channel. The other peaks in the UV channel represented impurities resulting from the standard purification process. The chromatograms show that ELSD is the better detection choice to analysis saikosaponins with less baseline noise and no interference from impurities. The peaks and the corresponding names of the saikosaponins are shown in table 2.

Peak	Compound
1	saikosaponin c, SSc
2	saikosaponin a, SSa
3	6''-O-acetylsaikosaponin a, 6''-acetylSSa
4	saikosaponin d, SSd
5	3''-O-acetylsaikosaponin d, 3''-acetyl-SSd
6	6''-O-acetylsaikosaponin d, 6''-acetyl-SSd

Table 2
Names of standard saikosaponins and corresponding peak numbers.

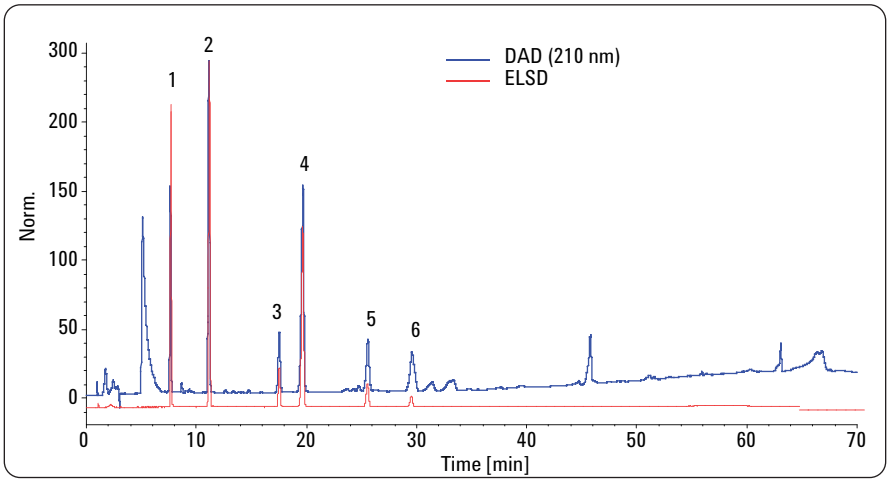

Figure 1
Chromatograms of six standard saikosaponins from conventional HPLC analysis using ELSD and UV detection.

Figure 2 shows the results from the analysis using the RRLC method. The run time was reduced from 60 to 6 minutes. The chromatograms showed flat baselines as well as good peak separation. The elution sequence was the same as in the conventional HPLC analysis because the two columns used the same packing material.

According to the China Pharmacopeia *B. bicaule* (sample 25) is not a typical Chaihu. However, in reality some TCM practitioners use this kind of *Buplerium*. Figure 3 shows the chromatograms from the ELSD and UV detection. The profile of sample 25 had more peaks than typical Chaihu when it was analyzed by HPLC. Comparing the ELSD and UV signals, two peak clusters were identified. This demonstrates that ELSD and UV detection are complementary on account of their ability to detect different kinds of compounds. The first cluster comprised compounds that were more volatile than solvent and contained chormophores. The compounds in the second cluster were less volatile than the solvent and contained no chromophores. The structures of these peaks are not yet know. Combining these results with a pharmacology study will reveal whether these peaks have any therapeutic effects.

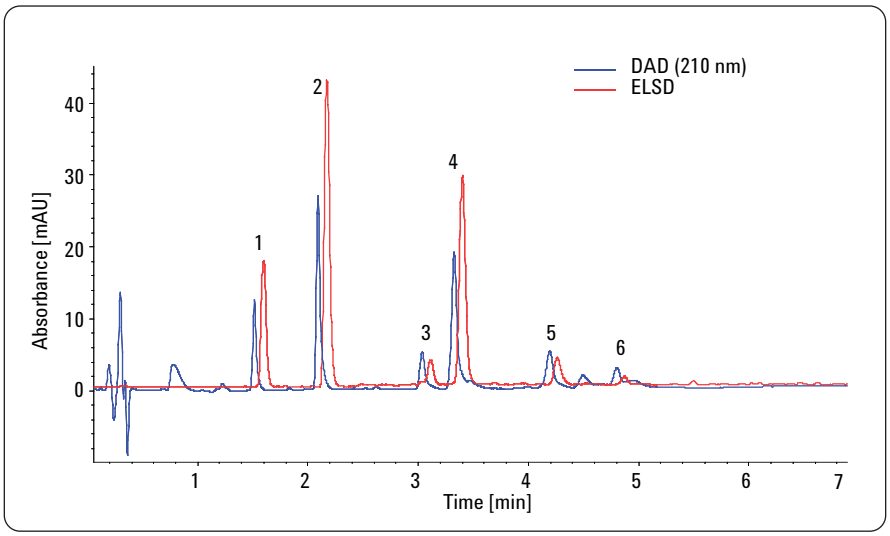

Figure 2
Chromatograms of six standard saikosaponins from RRLC analysis using ELSD and UV detection.

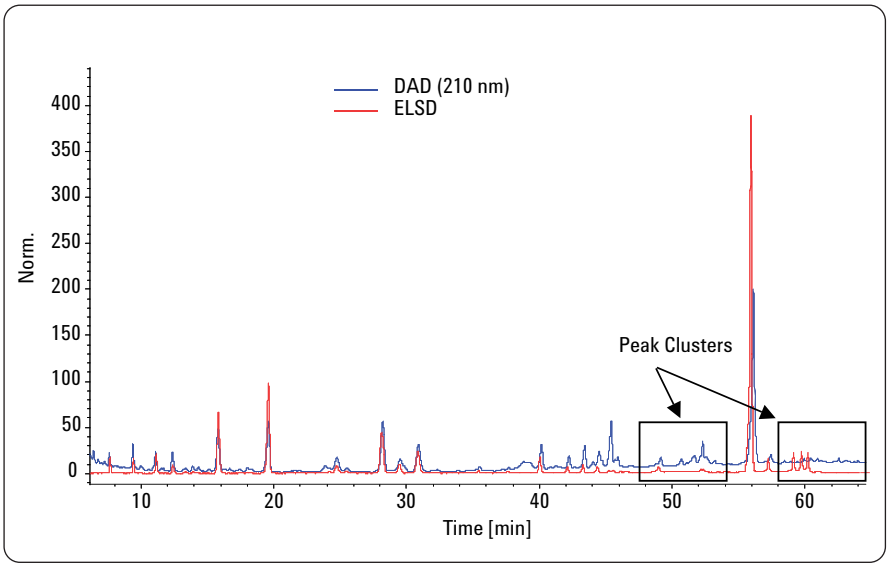

Figure 3
Chromatograms of Bupleunum bicaule (sample 25) from conventional HPLC analysis using ELSD and UV detection.

Figure 4 shows the results from the analysis of the same sample using the RRLC method. The run time was less than 14 minutes, which could give a fivefold increase in sample throughput. As with the conventional HPLC method, the stationary phase used the same chemistry thereby maintaining the same elution sequence and yielding a similar peak profile. In the following sections of this study, the RRLC method was deployed for the determination of the different TCM profiles in order to complete the analyses faster and to reduce costs.

As not all components exhibit the same behavior when using ELSD and UV detection, it is advantageous to equip the LC system with both detectors to monitor whether all analytes are eluted. This avoids non-detection of peaks and ensures no components are left in the column.

Figure 5 shows the chromatograms of four TCM samples from analysis using RRLC with ELSD detection. Samples 3 and 12 are Beichaihu and Nanchaihu respectively, which are described in the *China Pharmacopeia*. The difference between these samples is mainly in the last part of the profile. The saikosaponin profile was different from sample to sample. This made it difficult to analyze Chaihu from different origins when basing the measurement on the amount of saikosaponins only. Samples 21 and 25 had more peaks than the other samples as well as some common peaks. Samples 12 *(B. scorzonerifolium)* and 27 *(B. rockii)* have fewer peaks than the other samples in the later part of the elution.

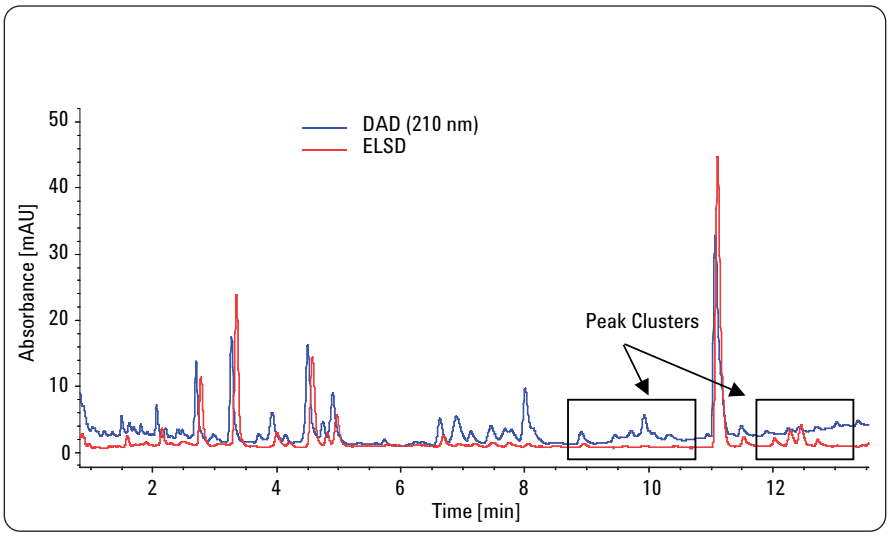

Figure 4
Chromatograms of Bupleunum bicaule (sample 25) from RRLC analysis using ELSD and UV detection.

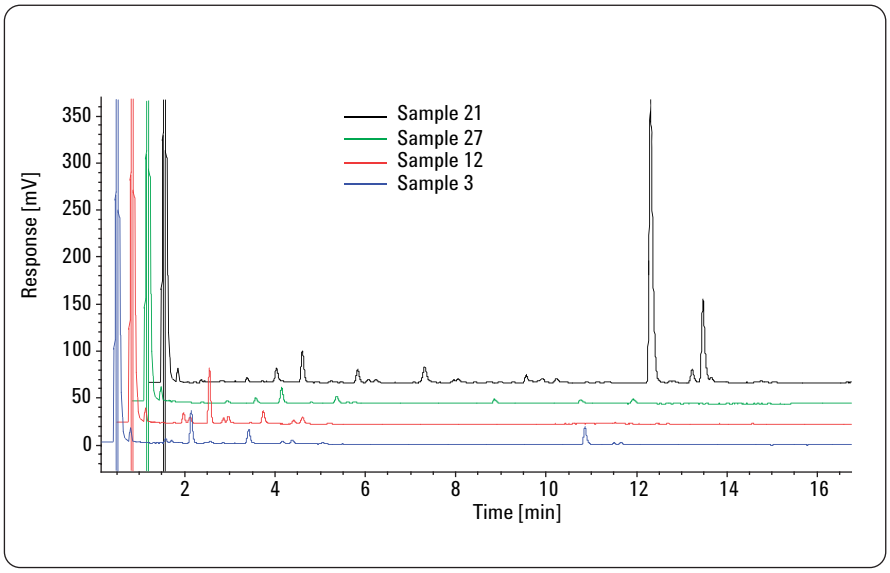

Figure 5
Chromatograms of four real-life TCM samples from RRLC analysis with ELSD detection.

The profiles of the different TCMs can help to make a first judgment as to the origin of the raw TCM. Currently, informatics research requires more samples to build a library for comparison. This will be part of future research work when more samples are available.

Quantitative analysis of the six saikosaponins

The RRLC system and method were used for quantitative analysis to reduce run times. ELSD detection is typically not linear and the calibration curve is dependent on sample and instrument parameters. Table 3 shows the result of quantitative analysis, whereby y refers to the log-transformed peak area, x to the log-transformed concentration of the reference saikosaponins (mg/mL). The limit of detection (LOD) is not exactly three times the concentration at the limit of quantification (LOQ) because the detector response is not linear for the analyzed saikosaponins. The different volatilities of samples and also the different particle sizes generated by the nebulizer meant that not all samples could be analyzed at maximum sensitivity under the selected conditions.

Based on the peaks areas of the real-life TCM samples, the concentrations of the different saikosapoinins are shown in table 4. The results show the different saikosaponin profiles in the TCMs of different origin. SSd and SSa are the main saikosaponins in all samples. Almost all samples contained SSc but not in such a high concentration as SSa and SSd. The other three saikosaponins were the decomposed components from SSa and SSd. This gave a quite different profile and some samples had no such decomposed saikosponins.

Compound (µg/mL)	Calibration curve*	r^2	Test range (µg/mL)	LOD (µg/mL)	LOQ
SSc	y = 1.5642x + 3.2933	0.9982	30–2000	10	30
SSa	y = 1.3892x + 3.0992	0.9967	33.5–3350	8.38	25.16
6''-acetyl-SSa	y = 1.8218x + 3.1273	0.9955	37.5–600	25	37.5
SSd	y = 1.4256x + 3.0996	0.9954	29–2900	14.5	29
3''-acetyl-SSd	y = 1.3251x + 2.8098	0.9976	30–2400	18	30

Table 3
Statistical analysis of the linear regression equation deployed in the determination of the six saikosaponins.
(* In the regression equation y = ax + b, y refers to the log-transformed peak area, x to the log-transformed concentration of the reference saikosaponins in mg/mL).

Compound	Sample 3 (µg/mL)	Sample 12 (µg/mL)	Sample 21 (µg/mL)	Sample 25 (µg/mL)	Sample 27 (µg/mL)
SSc	70	99	50	28	N/A
SSa	110	290	73	43	40
6''-acetyl-SSa	N/A	80	90	N/A	N/A
SSd	208	187	352	232	205
3''-acetyl-SSd	106	138	N/A	N/A	N/A
6''-acetyl-SSd	83	N/A	83	62	64

Table 4
Results of the quantitative analysis from the real-life TCM samples.
(N/A: below the detection limit)

Conclusion

The Agilent 1200 Series RRLC system is capable of running both conventional HPLC and RRLC methods for the quantitative analysis of saikosaponins. Whereas the conventional HPLC method required 70 minutes to achieve complete separation of all components in Chaihu, the RRLC method required only 15 minutes, thereby increasing sample throughput and reducing the cost per analysis. The Agilent 1200 Series ELSD was used for detection and the parameters were optimized so that good response was obtained for all saikosaponins. It was shown that ELSD is one of the best choices when analyzing compounds with no chromophores and that ELSD can provide a better signal when the compounds have weak chromophores as with saikosaponins. ELSD can also maintain better baseline stability when solvents are used that absorb in the low UV range.

Different peak profiles of Chaihu detected by ELSD and UV showed the necessity to use both detection techniques in serial because neither technique was capable of detecting all peaks when used individually. ELSD provide complementary fingerprint chromatograms when identification of the TCM origin was required. From the fingerprints of the TCM samples, the different profiles will help to judge the origin of TCMs. The methods described in this study application are applicable for quality control because quantitative analysis is possible.

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